T-CELL EPITOPE PEPTIDES

Technical Field

The present invention relates to T-cell epitope peptides of pollen allergen and a composition for peptide-based immunotherapy comprising the peptides as ~~an~~ effective ingredients. This composition is useful for treating and/or preventing pollinosis in springtime.

Background Art

About 10% of the Japanese population suffers from pollinosis developed in springtime such as cedar pollinosis. This condition has been on the increase and is attracting public attention.

The period when pollinosis is developed generally corresponds to the period when pollens scatter. In many cases, symptoms of pollinosis still remain after the season in which cedar pollens scatter because most patients with cedar pollinosis are also sensitized with Japanese cypress pollens (Hiroki cypress pollens) that start to scatter just after the cedar pollen-scattering period. Thus, patients who are also sensitive to Japanese cypress pollens suffer from the symptoms of pollinosis for a significant portion of the year.

Cedar pollens and Japanese cypress pollens possess common antigenicity (Takeshi Ide et al., Allergy Clinic 11, 174-178, 1991). The cross-reactivity of IgE antibodies between cedar pollens and Japanese cypress pollens has been established (Taniai M. et al., Mol. Immunol. 30, 183-189, 1993). The

positivity index of patients with spring pollinosis for their allergen-specific IgE antibodies is 83.5% for cedar pollens, 80.0% for Japanese cypress pollens, and 76.4% for both pollens (Mitsuhiro Okano et al., Allergy 43, 1179-1184, 1994). In addition, 60% of the patients with cedar pollinosis possess Japanese cypress pollen-specific IgE antibodies (Yozo Saito, Chiryo (Therapy) 78, 1571-1576, 1996). Based on these reports, it is generally recognized that cedar pollinosis patients can develop pollinosis to Japanese cypress pollens and vice versa.

Pollinosis is a typical immediate type I allergy induced by an antigen-antibody reaction between a pollen allergen (which is an antigen causing allergy and is substantially the same as an antigen) and an IgE antibody specific to the allergen. Thus, pollinosis is now prevented and treated using methods theoretically based on the mechanism by which type I allergics develop. This mechanism is briefly described below.

An antigen that has invaded the body is presented to helper T cells by antigen-presenting cells. As a result, B cells mature into antibody-producing cells. The antibody-producing cells produce an antigen-specific IgE antibody, which binds to the surface of mast cells. A subsequently ~~invaded~~ invading antigen binds to the IgE antibody on the mast cells. This stimulation releases chemical mediators like histamine from the mast cells, thereby causing an allergic symptom.

The following three methods are mainly used to prevent and treat allergies based on the above mechanism: 1) evasion of an antigen that causes allergy, 2) chemotherapy typically

using an anti-histaminic, and 3) desensitization therapy using an allergen. However, method 1) is difficult to implement practically, and method 2) is merely symptomatic therapy. Method 3) is expected to be the only treatment attacking the root problem, but it is not always effective and may ~~possibly~~ cause serious side effects such as anaphylactic shock.

For these reasons, peptide-based immunotherapy using T-cell epitope peptides of allergen has been recently attempted to prevent and treat allergies. T-cell epitopes participate in initiating and retaining an immune response to a protein allergen that causes clinical symptoms of allergies. These T-cell epitopes bind to HLA class II molecules on the surface of antigen-presenting cells to stimulate the related T-cell subpopulation. The stimulation is thought to trigger an initial response at the helper T-cell level. This initial response causes proliferation of T cells, secretion of lymphokines, a localized inflammatory response, migration of proliferated immune cells to the inflammatory sites, and activation of the B-cell cascade that precedes antibody production. IgE antibodies that are isotypes of these antibodies are critical to the development and retention of allergies. Furthermore, their production is influenced by the properties of lymphokines secreted by helper T cells at the beginning of the above-described cascade. The T-cell epitope is a basic element or the minimum unit to be recognized by a T-cell receptor. This epitope contains amino acid sequence necessary to recognize the receptor. Allergic inflammation

can be treated by controlling the response of the helper T cell, which plays a key role in immunosuppression, using the T-cell epitope peptide.

Known therapeutic agents for allergies using T-cell epitope peptides include a therapeutic composition comprising a T-cell epitope peptide of cat-origin allergen (a PCT application published in Japan (JP-WA) No. Hei 7-505365), a therapeutic composition comprising a T-cell epitope peptide of cedar pollen Cry j 1 (JP-WA-Hei 8-502163), and a multi-epitope peptide obtained by joining T-cell epitopes of cedar pollens Cry j 1 and Cry j 2 (Japanese Patent Application No. Hei 8-80702). The main allergen of Japanese cypress pollen, Cha o 1, is reported to have molecular weights of 45 KD or 50 KD. Each molecule has the same isoelectric point of 6.8 and consists of a protein containing 5% carbohydrate (Takeshi Ide, et al., Nippon Kafun Gakkaishi (Journal of the Japanese Pollen Association) 34, 39, 1988). However, their primary structures ~~are~~ were unknown, and accordingly, no T-cell epitope site has been identified on the allergen molecules yet. Recently, the present inventors succeeded in cloning the Japanese cypress pollen allergen gene, and clarified that, in addition to Cha o 1, another type of the allergen, Cha o 2, was present. Furthermore, the primary structures of Cha o 1 and Cha o 2 were determined (Japanese Patent Application No. Hei 6-335089).

Disclosure of the Invention

The period when cedar pollen scatter overlaps that of Japanese cypress pollen is referred to as the mixed

pollen-scattering period. These two pollens possess a common antigenicity, which makes it difficult to distinguish symptoms caused by cedar pollens from those caused by Japanese cypress pollens. The symptoms sometimes continue or develop even after the cedar pollen-scattering period. Since pollens found in the air during that period are mostly Japanese cypress pollens, these symptoms seem to be caused by Japanese cypress pollens. Since more Japanese cypress trees are planted than cedar trees, the amount of scattered Japanese cypress pollen is increasing year after year and will exceed that of cedar pollens in the near future. It is thus desirable to establish a method for preventing and treating allergies based on the root overall pollinosis caused by tree pollens in springtime, including Japanese cypress pollinosis and cedar pollinosis. Peptide-based immunotherapy using T-cell epitope peptides is expected to lead to allergy treatment based on the root pollinosis. As described above, several methods for such immunotherapy are known for cedar pollinosis. However, nothing has been reported on Japanese cypress pollinosis or on pollinosis caused by tree pollens in springtime, including cedar and Japanese cypress pollens.

An objective of the present invention is to provide T-cell epitope peptides useful for peptide-based immunotherapy for Japanese cypress pollinosis. Another objective of the present invention is to provide T-cell epitope peptides useful for peptide-based immunotherapy for patients with pollinosis caused by tree pollens in springtime including patients with

cedar pollinosis who show a cross-reactivity with Japanese cypress pollens.

The present inventors have identified a T-cell epitope site on the allergen molecules of Japanese cypress pollen by stimulating a T-cell line established from patients with Japanese cypress pollinosis with synthetic overlapping peptides that cover the entire primary structure of Japanese cypress pollen allergens, thus solving the above problems.

The present invention is comprised of the inventions described in each claim and will be described below in more detail.

The present inventors determined the amino acid sequence (described in Japanese Patent Application No. Hei 6-335089) of the major allergen, Cha o 1 (mature protein), of Japanese cypress pollen allergen shown as SEQ ID NO: 1 and that of Cha o 2 shown as SEQ ID NO: 2. The amino acid sequence of Cha o 1 has 80% homology to cedar pollen allergen Cry j 1, and that of Cha o 2 has 75% homology to cedar pollen allergen Cry j 2.

A number of amino acid substitutions are observed in the allergens derived from pollens, mites, and bee venom. These allergen species are called isoallergens. For example, eleven isoallergens have been isolated from birch tree pollen Bet v I, and their amino acid sequences differ from each other within a range of 2 to 15% (Swoboda, I. et al., J. Biol. Chem. 270: 2607-2613, 1995). At present, two isoallergens, in which six amino acid residues are substituted in a mature protein region, have been found in Cry j 2 (unexamined published Japanese Patent

Applications (JP-A) No. Hei 8-47392 and No. Hei 7-170986). One skilled in the art can reasonably expect that isoallergens would be present in Cha o 1 and Cha o 2 as well. Such isoallergens are also included in Cha o 1 and Cha o 2 referred to in the present invention.

The family of cedar trees is classified into nine genera, and the family of Japanese cypress, into seven genera. It is reported that allergens from Cryptomeria, Redwood, and Metasequoia, which belong to the cedar (Taxodiaceae) family, and Umbrella Pine, which is hypothesized to belong to either an independent family, the cedar family, or the pine family, show cross-reactivity ~~reactivity~~ with those from Japanese Cypress, Sawara Cypress, Oriental Arbor-vitae, Needle Juniper, and Chinese Juniper, which belong to the family of Cupressaceae (Takeshi Ide, et al., Allergy Clinic, 11, 174-178, 1991). In view of this report, cedar allergens are broadly cross-reactive with the allergens of Japanese cypress. Therefore, the peptides of the present invention are generally effective not only for Japanese cypress pollinosis but also for cedar pollinosis as well.

To obtain the T-cell epitope peptides of the present invention, overlapping peptides that cover the entire primary structures of Cha o 1 and Cha o 2 were synthesized; each peptide consists of the adequate number of amino acid residues (12 to 20 residues). The peptide of the present invention stimulates and/or suppresses the activity of T cells derived from patients with pollinosis caused by tree pollens in springtime. In other

words, the peptide of the present invention can induce proliferation of T cells or responses of T cells such as secretion of lymphokines, and/or can induce T-cell anergy (non-responsiveness). T-cell epitope sites on the allergen molecules can be identified using T-cell growth as an index in accordance with the method described in JP-A-Hei 8-47392. In particular, T-cell lines or T-cell clones, which are specifically reactive with Cha o 1 and Cha o 2, are established for every patient from peripheral lymphocytes of a patient with Japanese cypress pollinosis. The T-cell lines or T-cell clones are cultured in the presence of each peptide of the overlapping peptides. The epitope sites are identified by measuring the proliferation of T cells in the presence of the peptide (e.g., uptake of [^{3}H]thymidine into the cells) and calculating a stimulation index. The stimulation index (SI) used herein is obtained by dividing the radioactive level of [^{3}H]thymidine (cpm) taken up into the cells in the presence of the peptide by the level of [^{3}H]thymidine (cpm) taken up into the cells in the absence of the peptide (control). Based on the thus-obtained data, a mean stimulation index for each peptide is calculated for each patient group. The peptides found to induce T-cell response and/or induce T-cell anergy are defined as having a T-cell stimulating activity. The preferable T-cell epitope peptides of the present invention possess a T-cell stimulating activity and thus contain at least one T-cell epitope. Examples of the T-cell epitope peptide of Cha o 1 shown in Fig. 1 (specifically shown in Fig. 2, Fig. 3, and SEQ ID

NO: 3 through SEQ ID NO: 37) include Peptide #1-2 (SEQ ID NO: 4), Peptide #1-4 (SEQ ID NO: 6), Peptide #1-5 (SEQ ID NO: 7), Peptide #1-6 (SEQ ID NO: 8), Peptide #1-7 (SEQ ID NO: 9), Peptide #1-8 (SEQ ID NO: 10), Peptide #1-10 (SEQ ID NO: 12), Peptide #1-11 (SEQ ID NO: 13), Peptide #1-12 (SEQ ID NO: 14), Peptide #1-14 (SEQ ID NO: 16), Peptide #1-15 (SEQ ID NO: 17), Peptide #1-16 (SEQ ID NO: 18), Peptide #1-19 (SEQ ID NO: 21), Peptide #1-20 (SEQ ID NO: 22), Peptide #1-21 (SEQ ID NO: 23), Peptide #1-22 (SEQ ID NO: 24), Peptide #1-23 (SEQ ID NO: 25), Peptide #1-24 (SEQ ID NO: 26), Peptide #1-25 (SEQ ID NO: 27), Peptide #1-26 (SEQ ID NO: 28), Peptide #1-27 (SEQ ID NO: 29), Peptide #1-30 (SEQ ID NO: 32), Peptide #1-31 (SEQ ID NO: 33), Peptide #1-32 (SEQ ID NO: 34), Peptide #1-33 (SEQ ID NO: 35), and Peptide #1-34 (SEQ ID NO: 36) (Fig. 4). Examples of the T-cell epitope peptide of Cha o 2 shown in Fig. 5 (specifically shown in Fig. 6, Fig. 7, and SEQ ID NO: 38 through SEQ ID NO: 88) include Peptide #2-5 (SEQ ID NO: 42), Peptide #2-7 (SEQ ID NO: 44), Peptide #2-8 (SEQ ID NO: 45), Peptide #2-9 (SEQ ID NO: 46), Peptide #2-10 (SEQ ID NO: 47), Peptide #2-11 (SEQ ID NO: 48), Peptide #2-12 (SEQ ID NO: 49), Peptide #2-13 (SEQ ID NO: 50), Peptide #2-14 (SEQ ID NO: 51), Peptide #2-15 (SEQ ID NO: 52), Peptide #2-16 (SEQ ID NO: 53), Peptide #2-17 (SEQ ID NO: 54), Peptide #2-18 (SEQ ID NO: 55), Peptide #2-19 (SEQ ID NO: 56), Peptide #2-20 (SEQ ID NO: 57), Peptide #2-21 (SEQ ID NO: 58), Peptide #2-22 (SEQ ID NO: 59), Peptide #2-23 (SEQ ID NO: 60), Peptide #2-24 (SEQ ID NO: 61), Peptide #2-25 (SEQ ID NO: 62), Peptide #2-26 (SEQ ID NO: 63), Peptide #2-27 (SEQ ID NO: 64),

Peptide #2-30 (SEQ ID NO: 67), Peptide #2-31 (SEQ ID NO: 68), Peptide #2-32 (SEQ ID NO: 69), Peptide #2-33 (SEQ ID NO: 70), Peptide #2-34 (SEQ ID NO: 71), Peptide #2-35 (SEQ ID NO: 72), Peptide #2-36 (SEQ ID NO: 73), Peptide #2-37 (SEQ ID NO: 74), Peptide #2-38 (SEQ ID NO: 75), Peptide #2-40 (SEQ ID NO: 77), Peptide #2-41 (SEQ ID NO: 78), Peptide #2-42 (SEQ ID NO: 79), and Peptide #2-43 (SEQ ID NO: 80) (Fig. 8). More preferably, the T-cell epitope peptides have a mean stimulation index of 2.0 or more. Examples include Peptide #1-2 (SEQ ID NO: 4), Peptide #1-7 (SEQ ID NO: 9), Peptide #1-8 (SEQ ID NO: 10), Peptide #1-20 (SEQ ID NO: 22), Peptide #1-22 (SEQ ID NO: 24), Peptide #1-24 (SEQ ID NO: 26), Peptide #1-26 (SEQ ID NO: 28), Peptide #1-32 (SEQ ID NO: 34), Peptide #1-33 (SEQ ID NO: 35), and Peptide #1-34 (SEQ ID NO: 36), which are shown in Fig. 1, and Peptide #2-10 (SEQ ID NO: 47), Peptide #2-20 (SEQ ID NO: 57), Peptide #2-21 (SEQ ID NO: 58), Peptide #2-40 (SEQ ID NO: 77), Peptide #2-41 (SEQ ID NO: 78), Peptide #2-42 (SEQ ID NO: 79), and Peptide #2-43 (SEQ ID NO: 80), which are shown in Fig. 5. Most preferably, the T-cell epitope peptide has a minimum positivity index of 100. Examples thereof include Peptide #1-7 (SEQ ID NO: 9), Peptide #1-22 (SEQ ID NO: 24), Peptide #1-32 (SEQ ID NO: 34), and Peptide #1-33 (SEQ ID NO: 35), which are shown in Fig. 1, and Peptide #2-10 (SEQ ID NO: 47), Peptide #2-20 (SEQ ID NO: 57), Peptide #2-40 (SEQ ID NO: 77), Peptide #2-41 (SEQ ID NO: 78), Peptide #2-42 (SEQ ID NO: 79), and Peptide #2-43 (SEQ ID NO: 80), which are shown in Fig. 5. The "positivity index" used herein is obtained by multiplying a

mean stimulation index of a peptide by appearance frequency (%) of patients showing a T-cell response to the peptide.

To identify the epitope accurately, a peptide having the T-cell stimulating activity and thus containing at least one T-cell epitope may be modified by deleting any of the amino acid residues at the amino terminus or the carboxyl terminus of the peptide. The modified peptide may then be examined for any change in the T-cell stimulating activity. When two or more peptides that share the overlapping region exhibit the T-cell stimulating activity, a new T-cell epitope peptide containing all or part of the overlapping peptides is prepared, and its T-cell stimulating activity is measured in the same manner.

The T-cell epitope peptide of the present invention may ~~possibly~~ be immunologically associated with Cry j 1 or Cry j 2 in the T-cell cross-reactivity. Specifically, 1) the amino acid sequence of Cha o 1 has 80% homology to that of Cry j 1, and the amino acid sequence of Cha o 2 has 75% homology to that of Cry j 2; 2) the amino acid sequence of T-cell epitope peptide #1-2 of Cha o 1 (corresponding to ~~the amino acid sequence, SEQ ID NOS: 11-30~~, of mature type Cha o 1), which was identified in Example 5 of the present invention, is identical with the amino acid sequence of T-cell epitope peptide CJI-~~1~~2 of Cry j 1 (corresponding to ~~the amino acid sequence, SEQ ID NOS: 11-30~~ amino acids 11-30 of mature type Cry j 1; see Fig. 13 of JP-A-Hei 8-502163) except for two amino acid residues (Ala at position 12 of Cha o 1 corresponds to Ser of CJI-~~1~~2, and Asp at position 15 of Cha o 1 corresponds to Ala of CJI-~~1~~2); and 3) both cedar pollens and

Japanese cypress pollens have a common antigenicity. For these reasons, the origin of the T-cell epitope of the present invention is not limited to Japanese cypress. The T-cell epitope peptide of the present invention is effective not only for Japanese cypress pollinosis but also for cedar pollinosis.

In the T-cell epitope peptide of the present invention, the amino acid residues that participate in recognizing the T-cell receptor can be determined by a known method (for example, measuring the change in the T-cell stimulating activity which might occur due to the substitution of amino acid residues). The amino acid residues found to be essential for an interaction with the T-cell receptor are substituted with other amino acid residues to antigen-specifically control the T-cell stimulating activity so that allergic inflammation can be suppressed (increase the reactivity of T cells, alter the lymphokine-producing pattern, anergy etc.). It has been reported that, when one amino acid residue at the T-cell recognition site of the T-cell epitope peptide of cedar pollen Cry j 1 was substituted with another amino acid residue (substituting Thr at position 399 with Val) in a human allergy model, the resulting analog peptide showed substantially the same T-cell growth and IL-4 production as those of a wild type peptide, but showed increased production of IFN-γ that suppressed the production of IgE antibodies (Ikagawa, S. et al., J. Aller. Clin. Immunol. 97, 54-64, 1996). It has further been revealed that a binding motif of HLA class II molecules consists of three to five amino acid residues arranged via one

or two intermediary amino acid residues. When these residues consist of several kinds of specified amino acids, the peptide binds to the HLA class II molecules (Matsushita, S. et al., J. Exp. Med. 180: 877-883, 1994). Therefore, allergic inflammation can be prevented by determining the amino acid residues of the T-cell epitope peptide of the present invention, which are essential for the interaction with HLA class II molecules, by a known method, and substituting the ~~thus~~ relevant determined amino acid residues with other amino acid residues. Furthermore, the T-cell epitope peptide of the present invention can be modified so as to improve its solubility, thereby increasing its therapeutic or preventing effects or stability. Such modification includes substitution, deletion, and addition of the amino acid residues.

In the present invention, the T-cell epitope peptide preferably does not bind to IgE antibodies. Even if it binds to the IgE antibodies, the degree of binding is substantially lower than that of binding of the allergen of natural Japanese cypress pollens, from which the peptide is derived, to the antibodies.

The T-cell epitope peptide of the present invention preferably contains at least seven amino acid residues. These regions may be joined via a linker such as Arg-Arg or Lys-Lys that is sensitive to cleavage with an enzyme such as cathepsin or trypsin to enhance the sensitivity to processing by antigen-presenting cells. Thus, a peptide region can be produced to contain one or more T-cell epitopes. The T-cell

epitope peptide of the present invention may be used in combination with other peptides such as a T-cell epitope peptide of Cry j 1 (JP-WA-Hei 8-502163) and/or a T-cell epitope peptide of Cry j 2 (JP-WA-Hei 8-47392).

When a peptide containing at least one T-cell epitope peptide of the present invention is administered to an individual sensitive to Japanese cypress pollens and/or an individual sensitive to both Japanese cypress and cedar pollens, the peptide can control the individual's allergic response to the allergen(s). Such a peptide is thus effective for peptide-based immunotherapy. In particular, the T-cell epitope peptide of the present invention in combination with the T-cell epitope peptide of cedar pollen is more effective for peptide-based immunotherapy for a patient with pollinosis caused by tree pollens in springtime, represented by cedar and Japanese cypress pollens.

The T-cell epitope peptide of the present invention may be used as a diagnostic tool for pollinosis caused by Japanese cypress pollen allergens or other tree pollens that are immunologically cross-reactive with Japanese cypress pollen allergens. In such an application, the T-cell epitope peptide of the present invention is added to peripheral lymphocytes collected from a patient in an amount of about 0.1 μg/ml to about 1 mg/ml, and preferably about 1 to about 300 μg/ml. After the mixture is incubated for a week, uptake of [^{3}H]thymidine into the lymphocytes is assayed and assessed for diagnosis of pollinosis. The T-cell epitope peptide of the present

invention may also be used to evaluate either the function of T cells or proliferation of T cells or both ~~of them.~~

When the T-cell epitope peptide of the present invention is synthesized using recombinant DNA technology, host cells transformed with a nucleic acid containing a sequence coding for the peptide are cultured in a medium suitable for growing the host cells. The peptide can be harvested from the culture supernatant or from the host cells by a method known in the art. E. coli, yeasts, or mammal cells can be used as such host cells.

When the T-cell epitope peptide of the present invention is used in peptide-based immunotherapy for patients with pollinosis, the peptide may be administered together with pharmaceutically acceptable diluents or carriers. The "patient with pollinosis" as used herein includes patients with cedar pollinosis who show immunological cross-reactivity with the allergen of Japanese cypress pollen. The T-cell epitope peptide of the present invention can be administered in a simple manner, for example, by injection (subcutaneous or intravenous), instillation, rhinenchysis, oral administration, inhalation, or percutaneous administration. In the case of injection, a single dose of the peptide ranges preferably from about 1μg to about 30 mg, and more preferably from about 20 μg to about 10 mg.

Brief Description of the Drawings

Figure 1 shows T-cell epitope peptides of the Japanese cypress pollen allergen, Cha o 1, and a positivity index of

each peptide.

Figure 2 shows overlapping peptides (#1-1 to #1-28) of Cha o 1.

Figure 3 shows overlapping peptides (#1-29 to #1-35) of Cha o 1.

Figure 4 shows peptides containing T-cell epitopes of Cha o 1.

Figure 5 shows T-cell epitope peptides of Japanese cypress pollen allergen, Cha o 2, and a positivity index of each peptide.

Figure 6 shows overlapping peptides (#2-1 to #2-27) of Cha o 2.

Figure 7 shows overlapping peptides (#2-28 to #2-51) of Cha o 2.

Figure 8 shows peptides containing T-cell epitopes of Cha o 2.

Best Mode for Implementing the Invention

Examples of the present invention will be described below, but are not to be construed to limit the scope of the present invention.

Example 1

Synthesis of overlapping peptides

Based on the amino acid sequences of Japanese cypress pollen allergens Cha o 1 (SEQ ID NO: 1) and Cha o 2 (SEQ ID NO: 2), overlapping peptides consisting of 20 amino acid residues (14 residues in Peptide #1-35 (SEQ ID NO: 37) and Peptide #2-51 (SEQ ID NO: 88), each containing 10 overlapping

residues) were synthesized by the Fmoc method using a peptide synthesizer (PSSM-8, Shimadzu Seisakusho Ltd.). Thirty-five ~~kinds of~~ overlapping peptides were prepared for Cha o 1 (Fig. 1, SEQ ID NO: 3 through SEQ ID NO: 37), and 51 ~~kinds~~, for Cha o 2 (Fig. 5, SEQ ID NO: 38 through SEQ ID NO: 88). The ~~thus~~ synthesized peptides were all purified by high-performance liquid chromatography (HPLC) using an ODS column. The purity was 90% or higher in all of the peptides. The molecular weights of the purified peptides were identified by using a LASERMAT 2000 (Finnigan MAT Ltd.).

Example 2

Expression of the recombinant proteins in E. coli

Using a PCR technique, cDNA was amplified from plasmid DNA, in which Cha o 1 cDNA or Cha o 2 cDNA encoding a Japanese cypress pollen antigen had been cloned (Japanese Patent Application No. Hei 6-335089). A restriction enzyme recognition site was attached to the terminus of each cDNA. This DNA fragment was inserted into a histidine-tagged protein expression vector, pQE9, and the resulting vector was used to transform E. coli M15 (pREP4). Expression of the ~~gene~~ transforming ~~transformed~~ was confirmed for ampicillin-resistant clones by SDS-polyacrylamide gel electrophoresis. The protein expressed was purified using a Ni-NTA agarose affinity column.

Example 3

Establishment of T-cell line

A T-cell line on Cha o 1 was established as follows. Peripheral lymphocytes collected from 19 patients found

positive to Japanese cypress pollinosis using Ala STAT (Nippon DPC Corporation) or CAP-RAST (Pharmacia) were separated by specific gravity centrifugation using Ficoll-Paque. The lymphocytes (2 x 10^6 cells) were suspended in RPMI 1640 medium (GIBCO, Inc.) supplemented with 2 ml of plasma from the same patient (10%) or human AB type serum (20%, Banpoh Tsusho Co., Ltd.). The suspension was incubated ~~on~~ in a 24-well plate for 3 to 10 days (37°C, CO_2 incubator, TABAI, Inc.), together with 10 to 30 μg/ml of the recombinant Cha o 1 obtained in Example 2 or with a mixture of the overlapping peptides (0.01 to 1 μM) obtained in Example 1. When T cells activated by Cha o 1 stimulation were verified microscopically, 5 U/ml of IL-2 (Boehringer Mannheim) was added to the system, followed by incubation overnight. On the next day, the medium was replaced with fresh RPMI 1640 medium supplemented with 20 U/ml of IL-2, 10% or 20% human AB type serum. Incubation was continued for about 10 days with the medium being replaced every day in the same manner. The ~~T-cell line proliferated~~ resulting cell line was examined for its specificity, and a ~~part~~ sample of the T-cell line was frozen and stored. A T-cell line stimulated by Cha o 2 was also established from 20 patients with Japanese cypress pollinosis in the same way.

Example 4

Establishment of antigen-presenting cells

A lymphoblastoid cell line (B cell line) transformed by infecting B lymphocytes with EB virus (Epstein-Barr virus, EBV) to B lymphocytes was established to serve as antigen-presenting cells. First, EBV-producing B-95-8 cells (marmoset, ATCC CRL 1612) were

cultured in RPMI 1640 medium supplemented with 20% inactivated fetal calf serum (FCS, GIBCO Inc.). The culture supernatant was filtered through a 0.22μm sterile filter. The filtrate was frozen and stored at -80℃. Next, 1 ml of EBV solution was added to lymphocytes (2 x 10^6 cells) of a patient with Japanese cypress pollinosis, and the mixture was maintained at 37℃ for 30 minutes for infection. The EBV-infected cells were washed twice and then incubated for about 20 days in 20% FCS-RPMI 1640 medium supplemented with a final concentration of 200 ng/ml of Cyclosporin (Sandoz Pharmaceutical Co., Ltd.). After the cell mass was observable by the naked eye, incubation was continued in 20% FCS-RPMI 1640 medium for another 20 days. The resulting cells were frozen and stored until they were used.

Example 5

Identification of T-cell epitope peptide

The cultured B cell line established in Example 4 was treated with 50μg/ml of mitomycin C (Sandoz Pharmaceutical Co., Ltd.) for 30 minutes or exposed to an X ray (50 g ray), followed by washing four times with RPMI 1640 medium. After the B cells were inoculated on a 96-well plate (10,000 cells/well), the recombinant Cha o 1 or Cha o 2 was added thereto in a final concentration of 10 g/ml. To the control group was added a hemolytic streptococcus cell wall antigen (SCW) in a final concentration of 10μg/ml, Candida albicans antigen (CA) in a final concentration of 10μg/ml, and a Tuberculin antigen (PPD) in a final concentration of 1μg/ml). Subsequently, the T-cell line (20,000 cells/well) from the same patient, whose B

cell line had been established, was inoculated into each well. After a 48-hour incubation, 0.5μCi [3H]thymidine was added to each well, and incubation was continued for a further 16 hours. After the cells were collected on a glass filter using a cell harvester (Berthold), ~~an~~ uptake of [3H]thymidine into the cells was measured with a liquid scintillation counter to confirm the cell growth response.

After the T-cell line was confirmed to have proliferated specifically in response to Cha o 1 or Cha o 2, the growth response of the T-cell line to each of the overlapping peptides (final concentration of 1 μM) was examined in the same manner as above using the T-cell line established in Example 3. A mean stimulation index of the T-cell line in growth response to the overlapping peptides, an appearance frequency, and a positivity index calculated therefrom are shown in Figs. 1 and 5.

In addition, growth response of the T-cell line (N = 17) to modified sequences (SEQ ID NO: 89 and NO: 90) that corresponded to the amino acid sequences #2-11 and #2-12 in which one amino acid residue had been substituted, was examined. These two modified sequences exhibited T-cell stimulating activity of 1.6 and 1.2 in terms of the stimulation index, 16% and 11% in terms of the appearance frequency, and 25.6 and 13.2 in terms of the positivity index. As demonstrated above, the T-cell epitope peptide of the present invention retained its T-cell stimulating activity even when one or more amino acid residues were mutated, and the activity was enhanced in some

cases.

Industrial Applicability

The present invention provides peptides containing at least one T-cell epitope of Cha o 1 or Cha o 2, which are major allergens of Japanese cypress pollens. The present invention further includes a peptide fragment of other tree pollens showing immunological T-cell cross-reactivity with the peptides. These peptides are effective for peptide-based immunotherapy of pollinosis caused by tree pollens in springtime as represented by cedar and Japanese cypress pollens.